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THE GRANTS CLINIC*

Each issue of *Grants Magazine* carries an example of a "Winning Proposal," with a critique pointing out the strong points of the application. If the information is available, we also include an account of the preparation, planning, and pre-application negotiations that preceded the submission of the formal request. Effort is made to publish and analyze applications for projects in a wide variety of fields. Readers are invited to send in copies of applications that resulted in awards, from which examples will be selected for analysis and publication. Applications submitted for this feature should be send to The Grants Clinic editor:

Paul Hennessey, Vice President
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If requested, anonymity of the applicant and (or) the granting organization will be maintained. Analysts of these proposals will be selected for their special knowledge in the subject field.

The application reprinted here, "Differentiation and Physiology of a Neuroblastoma," is a request for support directed to the National Institutes of Health. George N. Eaves of the NIH is the critic and analyst of this "Winning Proposal." James M. Pike and Steven C. Bernard, both also of the NIH, comment on the budget forms accompanying the application.

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A SUCCESSFUL GRANT APPLICATION TO THE NATIONAL INSTITUTES OF HEALTH: CASE HISTORY*

by George N. Eaves

The peer review system used by the National Institutes of Health (NIH) and related variations used by a few other federal agencies have been described,[1-4] studied,[5,6] discussed,[7-10] investigated,[11] analyzed,[12-16] debated,[17] criticized,[18] ridiculed,[19] threatened,[20,21] attacked,[22] defended,[23] encouraged,[24] praised,[25] endorsed,[26-28] codified,[29] and legalized.[30] Although apparently nothing about the peer review system has been left unsaid, the preparation of a research-grant application still arouses uncertainty or apprehension in neophyte[31] and Nobelist scientist alike.[32] A few concerned people have tried to help by giving reasons for disapproval of applications,[2,33,34] by explaining the variables that influence approval or disapproval,[35] and by offering advice about preparation.[36-48] Nevertheless, as one of my astute colleagues† observed in relation to my own discussion of reasons for disapproval of applications,[2] it is easier to

*Address correspondence concerning the three papers that comprise THE GRANTS CLINIC to: Public Inquiries and Reports Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20014.

†The late Dr. Ilse Bry, who founded the *Mental Health Book Review Index*, which was published from 1956 to 1972, and who also served as Chairman of the Editorial Committee of that journal.

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identify the weaknesses in a proposal than to offer *specific* suggestions about how to write a successful application.

Having accepted the challenge from that observation, I soon concluded that it is probably not feasible to offer more specific practical guidance than already appears in the "Information and Instructions for Application for Research Grant, Form NIH (PHS) 398."* The alternative selected is to publish the text of a successful research grant application submitted to the NIH and allow those who read the proposal to draw their own conclusions about its merit.

In presenting only the narrative portion of the proposal, I have not meant to diminish the importance of other sections of a grant application, such as the budget, the facilities, and the biographical sketches. I have condensed into a footnote essentially all the information contained in the principal investigator's biographical sketch, except, of course, his publications, most of which are cited throughout the text of the proposal. The requested budget (not reprinted here) would now seem unrealistically modest in view of the inflation that has pervaded the national economy since 1970. I have therefore left the consideration of budgets, which also involves facilities, to the experts in grants management, two of whom have discussed this subject in the accompanying article.[49]

Criteria for Selection

The application published here fulfilled six criteria. First, I wanted the application to be the first submitted by an investigator who was initiating independent studies. Second, the text was not to exceed twenty pages, in keeping with a proposed restriction being considered currently by the NIH.[50] Third, the application had to have a priority score in the top 10 percent of all research grant applications reviewed at that meeting of the study section. Fourth, the primary factors used to judge the scientific and technical merit of the application had to be the same as those used currently.[29] Fifth, so that the proprietary rights of the principal investigator to his original ideas would be protected, the work proposed in the application had now to be completed. Sixth, the investigator had to have fulfilled the study section's expectations by successfully competing for renewal of the grant.

The application thus selected as a *case* of a successful, first application – not necessarily a model – was submitted to the NIH in 1970 by Dr. David R. Schubert* of The Salk Institute. The application, entitled "Differentiation and Physiology of a Neuroblastoma," resulted in the award of a three-year grant (NS-09658) from the National Institute of Neurological and Communicative Disorders and Stroke.

Modifications in the Application

In presenting the text of this application as a case history, I have refrained from imposing my own stylistic and organizational preferences on the application. In some instances, however, abbreviations that the author used only occasionally or that are now obsolete, such as BUdR for BrdU (5-bromodeoxyuridine), have been abandoned in favor of the terms they represent. No words within sentences, sentences within paragraphs, or paragraphs within sections have been rearranged; the content and order of the pro-

*The Instruction Sheet for NIH 398 (Revised 2-73) is included in the well-known package, "Application for Public Health Service Grant or Award," which is available in the business offices of institutions eligible for grants from the Public Health Service.

*Dr. Schubert, who was 26 years old when he wrote this proposal, was born in Indianapolis, Indiana. He received his B.A. degree in 1965 from Indiana University and his Ph.D. degree in 1969 from the University of California, San Diego, where he was a graduate student of Dr. Melvin Cohn. Dr. Schubert's postdoctoral research on neuroblastoma tissue culture was with Dr. Francois Jacob at the Institut Pasteur, in Paris, during the academic year 1969-1970, since when he has been a Member of The Salk Institute.

posal are exactly as originally submitted to the NIH. In three areas, however, I have introduced some modifications – mostly mechanical and nonverbal – that would either enhance the readability or increase the usefulness of the proposal. First, the introductory statement of the general objectives and the later statement of specific aims have been listed to give those who read the proposal[2] a readily comprehensible definition of the proposed work. Second, I have changed punctuation where necessary to effect editorial consistency and thus enhance readability. Since Dr. Schubert had already demonstrated his knowledge of the function of the series comma, which is not widely understood, even by many "editors" and teachers of English(!), this task required little effort. Third, I have modified the reference section so that it includes complete citations with titles of the articles and inclusive pagination, as recommended for scholarly publications,[51] for the benefit of the reader who may wish to know the precise subject of a cited paper or who may wish to request a photostatic copy from a library. In addition, Dr. Schubert has provided references to those papers that were either in press or in manuscript form at the time the proposal was submitted. Each of these references has been signaled with an asterisk that refers to an explanatory footnote. The intent in providing a complete reference section is to enhance the archival value of this proposal, which is an interesting account of the status of a specific aspect of cell and molecular biology eight years ago.

The proposal was selected without special consideration of its literary merits. Had the principal investigator's writing skills been seriously deficient, his scientific achievements probably would not have been as significant as they were at the time he wrote this proposal, and, consequently, this application would not have been among the successful.

The writing of most of us is not without flaws, and the successful applicant has not necessarily mastered the art of efficacious exposition. In some cases, the scientific originality and technical proficiency may be so meritorious as to be evident despite syntactical and grammatical improprieties and other weaknesses, but dependence on such good fortune is a frightfully risky situation and certainly incompatible with the attributes and goals of the scholar. As part of our professional growth and maturity, we must therefore continually try to improve our writing skills as well as all other aspects of our scholarship. Who is not dissatisfied with what he wrote eight years ago – or last week? Those who wish to concentrate on the grammatical and syntactical aspects of this proposal can profit from an analysis of the text in relation to the excellent advice recently offered by renowned experts.[47, 48]

In pointing out some of the features responsible for the success of the proposal, I have purposefully, and appropriately, restricted my comments to the opinions of the reviewing study section, since these are the only ones that count. A responsibility of the executive secretary of a study section is to obtain all the materials needed for the review of the application. For Dr. Schubert's proposal, no additional information was needed; his application was complete as submitted, and there was consequently no delay in processing it.

Review by Study Sections

The proposal to study the "Differentiation and Physiology of a Neuroblastoma" was reviewed by the Molecular Biology Study Section in September 1970, when I was the Executive Secretary of that Study Section. The proposed research was concerned with the differentiation of nerve cells and the chemical and electrophysiological properties of differentiating cells. The experimental system that was to be employed, as developed largely by Dr. Schubert, was unique: cells derived from a neuroblastoma that would grow in culture and differentiate when in serum-free media or when treated with 5-bromodeoxyuridine. From their preliminary investigations, the principal investigator and his

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colleagues had reported that these cells developed neuronal extensions with associated tubular and filament structures, nerve-specific enzymes, and electrophysiological properties similar to intact nervous tissue, including responsiveness to acetylcholine. Dr. Schubert proposed to continue his analysis of this interesting system by using the parent cell line in attempts to isolate mutants that affect developmental properties and electrophysiological behavior. Although the members of the Study Section readily recognized the overly ambitious scope of the project, they were favorably impressed. The résumé of the summary statement reads as follows:

> The proposed approach has great promise for advancing information about the regulation of animal cells. In fact, it may be the only way to obtain definitive answers. The system is one with great potential; it provides a ready means of invoking differentiation; and it would permit the correlation of biochemical, structural, and physiological parameters. In general, the experimental design is good, and the investigator appears to have a facile use of all techniques available. Although it is questionable whether "true" synapses could be developed in this system, there are so many questions that could be answered by the use of this system that the inability to obtain the formation of a true synapse would be inconsequential.

The detailed criticism included questions about the starting cell – a neoplastic neuroblast – not being a truly "wild-type" cell but a mutant with no guarantee for differentiation into a neuron. The reviewers also had doubts about formation in culture of clearly recognizable interneuronal synapses when the neuroblasts differentiated. The Study Section concluded, however, that:

> . . . this . . . interesting and ingenious proposal . . . describes the applicant as a skilled emigrant from molecular biology to the underdeveloped areas and their problems, although he may not know the terrain in sufficient detail to avoid illusions and misplaced effort. Nevertheless, the proposal represents a very interesting departure that definitely deserves the support necessary to insure its launching. Contact with reality, once the project is going, would take care of the rest.

Indeed when the continuation application was reviewed in April 1973, the Molecular Biology Study Section, which by then had changed significantly in membership, remarked that the confidence shown in the investigator almost three years before had been justified; he had been so productive as to warrant the accolades of "bright and imaginative" and "outstanding investigator." Continued support for four years was recommended with the same enthusiasm expressed earlier, even though the proposed approach remained questionable, since by then it was apparent that the tumors definitely did *not* form organized nervous tissue. Nevertheless, as in many research projects, the skills of the investigator and the other projects he proposed concurrently – in this instance, especially the prospects of new lines of nerve, muscle, and glial cells in clonal cell culture – offered the promise of a rewarding investment.

The next, and most recent, renewal application was reviewed in June 1977 by the Neurological Sciences Study Section because the research was now concerned primarily with the differentiation, physiology, and interactions of nerve, muscle, and glia. In reviewing progress during the previous period of support, the Study Section was pleased to note the large collection of cell lines from nerve, glia, smooth muscle, heart muscle, and skeletal muscle that had been isolated and subsequently characterized biochemically and electrophysiologically.

The report of this latest review clearly indicates one of the primary reasons for the investigator's success in the laboratory and in competing for later research grant funds: his ability to design original, imaginative, and useful experiments within the context of what he clearly and accurately recognized to be the strengths and limitations of the experimental system that he used. In the words of the reviewers:

> The most serious question that can be raised about this proposal is whether cell lines derived from neoplasms can generate useful information about mechanisms underlying cell-cell interactions. Certainly, if experiments are not carefully thought out and designed, this can be a problem. The refreshing aspect concerning Dr. Schubert's proposal is that he is constantly aware of the limitations of the system and designs his experiments accordingly.

As an interesting commentary on the objectivity of a system of peer review that involves collective judgment of merit, all of Dr. Schubert's research grant applications have been awarded about the same priority score[2] by each of the three reviewing Study Sections.

Acknowledgments

I wish to express my sincere appreciation to Dr. Schubert for permitting me to present his proposal as a case history. I would also commend him for his courage in doing so, because this application is likely to be reviewed critically by more people than have ever read any other proposal in the history of NIH granting!

I am also grateful for the gracious and generous help of Lois and Selma DeBakey, whose suggestions not only improved this article but also provided valuable instruction to their devoted student and friend.

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A PROPOSAL TO STUDY THE DIFFERENTIATION AND PHYSIOLOGY OF A NEUROBLASTOMA: A SUCCESSFUL RESEARCH GRANT APPLICATION SUBMITTED TO THE NATIONAL INSTITUTES OF HEALTH

by David R. Schubert, Ph.D.[1]

Abstract[2]

The differentiation and physiology of nervous tissue will be studied in vitro using closed tissue culture lines of a mouse neuroblastoma as a model system. The parameters of the differentiation process will be defined with emphasis on control mechanisms and the induction of specific cytoplasmic and membrane proteins. Mutants which are blocked at the various steps in the differentiation process will be isolated. Similarly, the molecular biology of the cholinergic receptor and catecholamine uptake, storage, and release will be defined by studying the wild-type functions in relation to a series of mutants blocked in these functions. Finally, the nature of synaptic specificity will be studied with respect to membrane structure in electrical and, perhaps, chemical synapses in the neuroblastoma and other cloned tissue culture lines.

[1] The Salk Institute, La Jolla, California.
[2] Page 2 of Form PHS-398.

Research Plan

A. Introduction

1. Objectives

The objectives are:

- to define, at the molecular level, the control processes involved in the differentiation of a neuroblast to a mature neuron;
- to understand the molecular biology of the coupling between cellular depolarization by acetylcholine resulting in the generation of an action potential and the subsequent release of catecholamine neurotransmitter in a sympathetic neuron; and
- to define the basis of neuronal and other intercellular specificities at the level of the membrane subunits involved in the contact.

2. Background

The analysis of differentiated function is hampered by the fact that the end cell usually does not divide. In the case of extremely heterogeneous cell populations such as those found in the endocrine, nervous, immune, and hepatic tissues, further advances require a way of establishing, in tissue culture, clones of end cells which express their differentiated phenotype. Such cells are most easily derived from neoplasms and have been successfully used in the study of endocrine (Sato and Yasumura, 1966), hepatic (Thompson, Tomkins, and Curran, 1966), and endoreticular (Cohn, 1967) cell function. In addition to using cloned, tissue-culture-adapted neoplasms to study a differentiated *function* such as antibody biosynthesis (Schubert, 1968), it has recently been possible to examine the *process* of differentiation in a mouse neuroblastoma (Schubert, Humphreys, Baroni, and Cohn, 1969; Schubert, Humphreys, de Vitry, and Jacob, 1971; Schubert and Jacob, 1970).

The experimental approach that was used during our investigation of antibody biosynthesis and assembly in mouse plasma cell tumors was somewhat novel in the field of animal cell research, but classical in the methodology of molecular biology. The wild-type function of immunoglobulin synthesis was defined (Schubert, 1968; Schubert, 1970), and a series of mutants defective in this function was isolated (Schubert, Munro, and Ohno, 1968; Schubert and Horibata, 1968). An analysis of these mutants revealed that they were blocked at various stages of immunoglobulin biosynthesis and assembly (Schubert and Cohn, 1968).

The characterization of the mutant cell lines shed a great deal of light on the wild-type synthetic mechanisms and generated a number of facts and concepts which would have been missed if only the wild-type cell lines were studied (Schubert and Cohn, 1968; Schubert, 1970; Schubert and Cohn, 1970). It is evident that this approach to the study of mammalian cell function should be extended to other types of differentiated end cells and also to the process of differentiation itself. Such studies would not only extend our present knowledge and verify our predictions; they could also define new concepts which are not readily deduced from investigations of the normal function. If coupled with somatic cell genetics, an enormous amount of information could be gained about regulatory mechanisms in higher organisms.

It has recently been possible to induce a cloned neuroblastoma tissue culture line to differentiate into a population of cells which has the morphological and functional characteristics of mature neurons (Schubert, Humphreys, Baroni, and Cohn, 1969). The process of differentiation is defined in the following manner. When grown in suspension culture in bacteriological Petri dishes, the cells have a primitive round cell morphology – high nuclear cytoplasmic ratio and no morphological specialization; however, when given a surface on which to attach, such as glass or collagen, cells send out processes (neurites) up to 3 mm in length and assume the morphology of mature neurons. Whereas the cells grown in suspension are not, the attached cells are stained by the Bodian silver procedure for neurons. Electron microscopy reveals that the attached cells contain

neurofilaments, neurotubules, and dense-core vesicles indicative of nerve fibers. Both free floating and attached cells have tyrosine hydroxylase activity characteristic of sympathetic nervous tissue (Schubert, Humphreys, Baroni, and Cohn, 1969).

By recording from single cells, it has been shown that there is a sequential development of resting potential and acetylcholine sensitivity concomitant with morphological differentiation (Harris and Dennis, 1970). The highly differentiated end cells (attached cells with long cytoplasmic processes and well developed neurotubules) have resting potentials of about –60 mV and are induced to generate 85 mV action potentials by current depolarization or the extracellular (iontophoretic) application of acetylcholine. The undifferentiated round cells growing in suspension culture have much lower resting potentials but are also depolarized by acetylcholine. The spread of acetylcholine sensitivity over the cell surface during maturation was shown to be similar to that of naturally developing nervous tissue, and the existence of electrical synapses between cells via cell processes has been demonstrated. Electron microscopy has revealed process endings containing high concentrations of mitochondria and dense-core vesicles which are embedded in soma and processes of other cells (Schubert, Humphreys, Baroni, and Cohn, 1969). Such nerve endings are usually associated with chemical synapses, but the physiological demonstration of chemical transmission is still lacking.

In a continuation of the above work, conditions have been defined in which *quantitative* differentiation of neuroblast to neurons can be *induced* within 24 hours (Schubert, Humphreys, de Vitry, and Jacob, 1971). This process is completely reversible, and it has been shown that the synthesis of a limited number of proteins can be either induced or repressed, depending on the growth conditions of the cells and their state of differentiation (Schubert, Humphreys, de Vitry, and Jacob, 1971). We feel that this experimental situation offers a unique opportunity to examine the control mechanisms at the molecular level in a reversible differentiation process which is quite analogous to in vivo neuronal development and regeneration (Harris and Dennis, 1970).

Since this tissue culture line can be depolarized to generate an action potential by acetylcholine, and since it contains the catecholamine neurotransmitters (Schubert, Humphreys, Baroni, and Cohn, 1969; Agusti-Tocco and Sato, 1969) characteristic of postganglionic sympathetic neurons, it follows that this neuroblastoma cell line is ideally suited for both the study of the acetylcholine receptor and the interaction between receptor and membrane which leads to depolarization, propagation of the action potential, and the subsequent release of transmitter from pre-synaptic cells. At present, virtually nothing is known about the chemistry of this linkage.

In addition, it has been shown that the neuroblastoma cell line can form electrical synapses in vitro (Harris and Dennis, 1970) through contacts which have some morphological semblance to chemical synapses (Schubert, Humphreys, Baroni, and Cohn, 1969). The neuroblastoma also formed similar contacts with a muscle tissue culture line (Schubert, unpublished observation). It should thus be possible to study the nature of the specificity involved in these synaptic contacts and the effect of the innervated end cell on nerve development, and, conversely, the effect of innervation on muscle development.

3. Rationale

Wild-type function will be studied in detail, and mutants will be isolated which are blocked in these functions. An analysis of the sites of the mutational blocks will help define the wild-type function. This is clearly the classical approach to complex biochemical mechanisms, and perhaps the *only* approach which can lead to clear answers. Surprisingly, however, this approach has not until recently been used to study a differentiated function in animal cell tissue culture lines (Schubert, Munro, and Ohno, 1968; Schubert and Horibata, 1968; Schubert and Cohn, 1968; Schubert and Cohn, 1970).

B. Specific Aims

The first aim is to define the molecular control processes involved in the differentiation of a neuroblast to neuron in terms of the induction and repression of specific proteins, the level at which these control mechanisms act, and the process by which the cell manipulates what appears to be a surface stimulus that induces differentiation. This study will be supplemented by the isolation of mutants which are unable to differentiate.

The second aim is to determine the physical-chemical nature of the acetylcholine receptor and the mechanism by which acetylcholine depolarization is coupled to the release of catecholamine neurotransmitters. Again, these mechanisms will be studied through the use of mutants blocked at the receptor site, the sodium-potassium-adenosine-triphosphatase-linked pump, and catecholamine uptake and storage.

The third aim is to isolate, purify, and characterize the membrane components involved in the specificity of cell-cell interactions – both in the nervous system and between cells of different tissue origin.

C. Methods

I. Differentiation

Since differentiation of the neuroblastoma can be induced by transferring cells grown in suspension culture to culture dishes where they can attach to a surface, it follows that the stimulus leading to induction is associated with the interaction between that surface and the limiting membrane of the cell. Analogous membrane-mediated cell-cell and cell-effector interactions are possibly the most important, yet least understood, group of reactions involved in cellular and tissue differentiation. By looking at the induced differentiation of the neuroblast, it should be possible to define the role of the cytoplasmic membrane in these processes.

The induction process has been clearly defined as an interaction (presumably electrostatic) between the cell and the surface of the tissue culture dish (Schubert, Humphreys, de Vitry, and Jacob, 1971). Morphological differentiation can be quantitatively induced within one day by placing cells in tissue culture dishes in the absence of serum. This process can be reversed by compounds which buffer the cell-surface interaction, such as nonspecific proteins and serum. In the presence of serum, *partial* differentiation of the culture takes about five days, and it has been shown that this process is controlled by a low-molecular-weight metabolite secreted from the cells, which enhances the electrostatic interaction between the cell and surface, thus leading to induction. Treating the cells with 5-bromodeoxyuridine (10^{-4} to 10^{-7} M) will mimic the effect of the low-molecular-weight fraction (Schubert and Jacob, 1970).

Differentiation is not coupled to cell division, for cell division can be inhibited without the concomitant induction of differentiation, and, conversely, differentiation can be induced in an exponentially growing culture (Schubert, Humphreys, de Vitry, and Jacob, 1971).

The induced differentiation of the neuroblastoma also *appears* to be an example of translational control in animal cells, for differentiation can be blocked with protein synthesis inhibitors such as puromycin and cycloheximide, but not with concentrations of actinomycin D which inhibit RNA synthesis greater than 90 percent (Schubert, Humphreys, de Vitry, and Jacob, 1971). The chemistry of this process remains to be defined.

(a) Isolation of Mutants. Since the differentiation of the neuroblastoma requires a surface on which the cells can attach, it follows that a surface-mediated stimulus may be necessary, but perhaps not sufficient, to induce differentiation. Two distinct processes, one leading to cell attachment and the other to differentiation, could be dissociated by the selection of mutants blocked in the ability to attach to a surface and those which attach but do not differentiate. Both types of cells could easily be selected following muta-

genesis and expression for several generations by plating cell suspensions under conditions where there is 100 percent attachment and differentiation (Schubert, Humphreys, de Vitry, and Jacob, 1971). The cells unable to attach are selected by removing the culture fluid, allowing the decanted, unattached cells to grow up in suspension, and then repeating the selection procedure against attached cells. After several cycles of selection, the resultant cells would be cloned in agar (Schubert, Humphreys, Baroni, and Cohn, 1969). Each clone would then be individually tested for its ability to attach and differentiate.

The second class of differentiation-defective mutants would be those cells which attach but do not differentiate in the sense that neurite formation is lacking. The isolation of these mutants would require the picking of individual cells by micromanipulation (Horibata and Harris, 1970) or by trypsinization of isolated cells. Clones of these cells would be grown up and again tested for their ability to attach and differentiate.

(b) Characterization of Mutants. Mutants which do not attach, or those which attach and do not differentiate, will be initially screened on different substrates which normally lead to attachment. For example, mutants isolated on tissue culture plates will be screened for attachment and differentiation on collagen and glass. If there are differences, the nature of the stimulus leading to induction of differentiation could be studied. In any case, the mutational defects could be defined in the following terms.

There are three groups of mutants which one would expect to find. The first would be cells defective in some substance required for attachment. This function is not *necessarily* related to induction but is only a prerequisite. Examples of this class could be more carefully characterized by looking for differences between them and the wild type in the various cytoplasmic membrane components.

A second class would be those cells which can attach but have lost the specific function – presumably defined in terms of a membrane protein – which serves as a transducer between the extracellular phenomenon of attachment and the intracellular control of differentiation. Again, differences in membrane structure could be examined, the study being greatly facilitated by comparison with the wild-type cell line.

The third class of mutants, which are phenotypically similar to the second, is that which attaches but is defective in some cytoplasmic process required for differentiation. For example, it is known that the undifferentiated round cells lack neurotubules, while the differentiated attached cells contain large quantities of these organelles (Schubert, Humphreys, Baroni, and Cohn, 1969). It has also been shown that the spectrum of proteins synthesized by the round and differentiated cells is quite different as defined by acrylamide electrophoresis (Schubert, Humphreys, de Vitry, and Jacob, 1971). It follows that a structural defect in one of these proteins may inhibit differentiation.

One such defect may be the inability to synthesize or assemble the neurotubule protein which probably is responsible for the structural stability of neuronal processes. The purification of the neurotubule protein is quite easy (Marantz, Ventilla, and Shelanski, 1969). Once the protein is purified, antisera could be prepared against it and the serum used as an assay for the neurotubule synthesis in a manner analogous to the use of antisera to detect immunoglobulin biosynthesis (Schubert, 1968). In addition, antisera could be used to study the assembly of the neurotubule organelle as with immunoglobulin assembly (Schubert, 1968). Since the synthesis and assembly of the microtubule organelle is inducible with differentiation (Schubert, Humphreys, Baroni, and Cohn, 1969; Schubert, Humphreys, de Vitry, and Jacob, 1971), this type of investigation is of particular interest for three reasons. First, the control mechanisms involved in microtubule synthesis could be studied in relation to the process of differentiation. For example, what is the relationship between cell attachment and induction of specific protein synthesis? Secondly, it could be asked how the microtubule is assembled in the cell. The fact that there may be de novo protein synthesis

makes this question easier to answer than with other systems. Thirdly, mutant cell lines which attach and do not differentiate will be available. It is possible that some of these cells will be blocked at a step in microtubule synthesis. By using antisera selection techniques (to be discussed later) it may be possible to select differentiated cell lines which lack microtubules. Information of the above type cannot help but lead to the definition of a function for the cellular microtubules.

A number of other types of experiments may also lead to a better understanding of neuroblast differentiation. For instance, it is known that mitotic inhibitors such as colchicine also inhibit microtubule formation (Adelman, Borisy, Shelanski, Weisenberg, and Taylor, 1968) and axonal flow (Kreutzberg, 1969). In addition, by the use of such inhibitors of microtubule formation it may be possible to define the function of the microtubules in the following way. It is known that during the development of axons, the axoplasm at first lacks microtubules, which then appear to develop as the cell matures (Schubert, Humphreys, Baroni, and Cohn, 1969). If cells were allowed to attach and initiate differentiation, followed by the addition of colchicine, then cells may develop without microtubules. Correlated electrophysiology and electron microscopy could then answer questions relative to microtubular function, such as: what is the effect on resting and action potentials, are mitochondria and synaptic vesicles localized in axoplasm as before, and are the cells lacking microtubules able to form functional synapses as untreated cells? By the use of isotopically labeled cells and antisera directed against microtubule protein, it should also be possible to define the mode of action of colchicine: where in the synthesis and assembly of microtubules does this compound act?

As mentioned previously, it has been shown that micromolar amounts of 5-bromodeoxyuridine can rapidly *induce* differentiation of the neuroblastoma under conditions which normally inhibit this process (Schubert, Humphreys, de Vitry, and Jacob, 1971; Schubert and Jacob, 1970). Although of doubtful physiological significance, this observation is of some interest since, in all other systems examined, 5-bromodeoxyuridine *inhibits* the expression of the differentiated trait. (See, for example, Bischoff and Holtzer, 1970.) It has repeatedly been argued that this inhibition of differentiation effects specifically the transcription of genes involved in differentiation (Bischoff and Holtzer, 1970). In light of the above results, however, this is an unlikely proposal, for the primary site of action of 5-bromodeoxyuridine is probably the membrane, which, in turn, alters the metabolism of the cell *through* its increased affinity for the surface of the tissue culture dish. If this hypothesis is true, it should be possible to induce differentiation with 5-bromodeoxyuridine under conditions where cell division and DNA replication are inhibited – with cytosine arabinoside or ultraviolet light. It may also be possible to detect surface antigens which are unique to the 5-bromodeoxyuridine-induced cells and different from the normal differentiated and undifferentiated cells. The electrophysiological properties and distribution of acetylcholine sensitivity on the cells will also be examined for the possibility of finding a unique state of membrane development which can be analyzed chemically and which will shed some light on the natural process.

It has also been observed that the chromosome ploidy of the neuroblastoma varies as a function of its differentiated state. The undifferentiated cell line is tetraploid, while the majority of the differentiated cells are octaploid or greater (Schubert, Humphreys, Baroni, and Cohn, 1969). Since the differentiation of these cells is reversible (Schubert, Humphreys, de Vitry, and Jacob, 1971), this poses an interesting problem in the control of mitosis and its relationship to cell division. Although it has been shown that cessation of mitosis does not *induce* differentiation and that the differentiated cell can dedifferentiate and divide, the detailed relationship between the increase in ploidy and differentiation has not been examined. This could be readily accomplished by a combination of karyotyping,

DNA trace labeling with thymidine, radioautography, and pulse-chase experiments. (See, for example, Bischoff and Holtzer, 1970.) Another approach will be to look at surface antigens unique to differentiated cells, the precursor round cell, and the 5-bromodeoxyuridine-induced differentiated cells. Does attachment induce the synthesis on new surface antigens, and are there unique antigens which define two states? Once surface antigens are defined with antisera, it should be possible to isolate these structures and characterize their role in the differentiation process. For example, if the formation of an antigen unique to the differentiated cell is blocked under normal condition of differentiation, are the cells able to attach to the surface, or attach but not differentiate? It may be possible to select against a given surface antigen and find mutants which are blocked at some stage in the differentiation process. The pertinent technology for these experiments will be discussed later (Sections II and IV).

II. Junctional Specificity

(a) Nervous Tissue. Although electrophysiological evidence for chemical synapses is still lacking, morphological evidence indicates that this type of cell contact may be present (Schubert, Humphreys, Baroni, and Cohn, 1969). Electrical contacts via cell processes, which may be functional precursors to chemical synapses, have been observed (Harris and Dennis, 1970). In addition, there is morphological evidence for synapses between the neuroblastoma and a myoblast tissue culture line (Schubert, unpublished observation). It thus appears that the neuroblastoma tissue culture line has the potential for forming functional contacts with a variety of target cells, although sympathetic neurons usually do not, in vivo, form cholinergic-like synapses where direct cell-cell contact is involved (Iverson, 1967). These results may be analogous to the specification of axons by their motor and sensory end organs (reviewed by Edds, 1967).

Since the specificity of nervous contacts is probably defined in terms of complementary membrane proteins at the synaptic site, and since the amount of membrane surface area involved in the synapse is quite small relative to the total area of the membrane, it follows that the characterization of the proteins involved in specificity is technically impossible unless these proteins are amplified in some manner. There are two experimental approaches to this problem. One involves the purification of presynaptic nerve terminals or synaptosomes (Whittaker and Sheridan, 1965) and the other involves the use of antisera directed against *unique* surface components. Perhaps the combined use of these techniques could help characterize the proteins involved in synapse formation – a somewhat hopeless task when whole animal tissue is used. The following paragraphs outline one of several possible experimental approaches.

Cells would be allowed to differentiate either on different surfaces, such as glass, collagen, and sponge, or in the presence of different types of cells, such as embryonic smooth- and skeletal-muscle tissue culture lines. Once contacts are made and demonstrated morphologically and electrophysiologically, synaptosomes would be prepared, the synaptic vesicles, limiting membranes, and mitochondria separated by lysis in hypotonic buffer and differential centrifugation (Whittaker and Sheridan, 1965), and antisera prepared against each preparation. In the case of cells which were grown on different surfaces, synapses would be formed between like cells, as would be some of the contacts in the mixed populations. Once made, the antisera would be absorbed with membrane preparations from heterologous cell types and also synaptosome membranes of the neuroblastoma-neuroblastoma type. The resulting antisera should be specific for any unique proteins found in each preparation, which presumably would define the specificity of the synapse. Once this assay for these membrane proteins is available, it would be possible to purify and characterize them from large quantities of cells. By labeling cells during the differentiation pro-

cess with amino acids, it could be shown when in the maturation process (Harris and Dennis, 1970) these proteins are synthesized. A great deal of information could be collected about their structure simply through the use of isotopic labeling, serological precipitation, and examination of the labeled proteins dissociated from the precipitate (Schubert, 1968), without having to prepare large numbers of cells. Similarly, by preparing antisera against cells at different stages of differentiation, it may be possible to define these stages in terms of cell-surface structure which, in turn, may be related to specificity.

It is not known whether the cloned neuroblastoma round-cell stem line is generating round cells which, when induced to differentiate, give rise to cells with different potential specificities, or whether all of the cells derived from this clone are equipotential with regard to junctional specificity. There is, however, good evidence that different clones derived from the same mouse tumor, and also individidual cells derived from the same clone, differ in their electrical properties (A. J. Harris, personal communication).

In addition, a number of different clones from the round-cell population and the original tumor will be isolated and characterized with respect to surface antigens, electrical properties, and the potentiality for differentiation and synapse formation. Similarly, it would be most interesting to clone the attached, differentiated cells and ask how their morphological, electrical, and antigenic properties differ from other cells derived from the same clone.

(b) Tight Junctions in Other Tissue Culture Lines. Electrical or "tight" junctions have been demonstrated both in the central nervous system (Bennett, Nokajima, and Pappas, 1967) and between cells of most other tissues (Loewenstein, 1967), including embryonic (Potter, Furshpan, and Lennox, 1966). It has been strongly argued that these permeability junctions play an essential role in development and nervous function (Loewenstein, 1967), yet little is known about their genesis and mechanism of specificity. Since similar electrical junctions have been demonstrated in fibroblast tissue culture lines (E. S. Lennox, personal communication), it follows that the study of tight junctions in various cloned tissue culture lines could lead to an understanding of the mechanism of tissue-tissue interactions and also serve as a model system for nervous specification. This is particularly true since tight junctions have both functional (Politoff, Socolar, and Loewenstein, 1967) and structural (Brightman and Reese, 1969) similarities to chemical synapses. In addition, it is relatively easy to isolate pure sheets of tight junctional membrane (Benedetti and Emmelot, 1968), and it has been demonstrated that this material is made up of *hexagonal arrays of subunits* (Benedetti and Emmelot, 1968). That such a repeating subunit structure is involved in specific cell-cell interactions is predictable from our knowledge of enzyme structure but has not been studied at the molecular level. The following paragraphs outline an experimental approach to the mechanisms involved in the specificity and also the development of cell-cell contacts.

To define the mechanism of specificity, the limiting membranes of a large quantity of cloned contact-inhibited cells such as 3T3 fibroblasts, where electrical coupling has been demonstrated (Section III), will be isolated. Tight junctional membranes will be isolated according to Benedetti and Emmelot (1968) and checked for purity by electron microscopy.

Once purified, the complex will be dissociated under as mild conditions as possible, such as high salt and low pH (Cook and Koshland, 1969), until a subunit of the approximate size of that observed by electron microscopy is generated and characterized by density centrifugation and electron microscopy. Then, conditions will be defined where specific reaggregation can be demonstrated, again using the technique of density gradient centrifugation.

Reaggregation could be quantified in a manner analogous to that used to study the complementation between immunoglobulin subunits (Mannik, 1967; Schubert and Cohn, 1968). By isolating tight-junction subunits from several cloned tis-

sue culture lines and assaying the relative competition between homologous and heterologous subunits, the basis of tissue specificity may be elucidated. The physico-chemical nature of the individual subunits would then be determined and the structural proteins isolated. Specific questions could be asked regarding the structure-function relationship between the proteins (or glycoproteins) making up the subunit. For example, are there a number of proteins shared by all of the mouse cells, while a few are different? What is the relationship between the tight-junction proteins and those involved in nerve specificity? [See Section II(a).]

To examine the natural distribution of the tight-junction determinants and to establish when in the growth of tissue culture cells these determinants are expressed, antisera will be prepared against the purified tight-junction membrane sheets and also against the subunits and component molecules at each stage of purification. By adsorption of the sera with membranes from cells functionally lacking tight junctions, specific antisera could be prepared against the junctional determinants. These antisera could then be used to define the time of induction of these surface proteins – is it before or after cell-cell contact? – by the use of radioactive labeling and indirect immune precipitation (Schubert, 1968) and the cytotoxic assay for surface antigens. As with synaptosomal proteins [Section II(a)], specific antisera could be prepared against tight junctions from several mouse tissue culture lines and used to define the molecular determinants of tissue specificity. In addition, the role of the tight junction in the establishment and release from contact inhibition could be studied once some insight has been gained into the structure and specificity of the synapse. Clearly, the problems surrounding cell-cell interactions such as contact inhibition and tissue specification are central to cancer research, but as yet these problems have, for the most part, remained unsolved.

III. Electrophysiology and an Alternative Assay for Membrane Depolarization

The development of the electrical properties of the neuroblastoma has been studied in some detail, particularly with respect to the spread of acetylcholine sensitivity during cytodifferentiation (Harris and Dennis, 1970). Harris will continue the work that he originally initiated at The Salk Institute. This will include more work on the development of acetylcholine sensitivity in the neuroblastoma (to be correlated with the biochemistry of the receptor), examination of cells for evidence of chemical and electrical synapses, and the characterization of mutant cell lines with respect to their electrophysiological properties.

Since electrophysiological recording from single cells is a rather difficult and tedious procedure, attemps will be made to develop an alternative procedure for the measurement of membrane permeability changes. Clearly, the most promising of these is the measurement of radioactive potassium or sodium uptake and release with whole cells or closed membrane fragments. The techniques using whole cells have been worked out for the red cell system (Villamil and Kleeman, 1969) and could easily be adapted to the neuroblastoma system. Several modifications, such as the collection of cells on Millipore filters instead of by centrifugation, would be introduced. It may also be necessary to inactivate the adenosine triphosphatase-linked sodium-potassium pump with ouabain before the technique is sensitive enough to detect acetylcholine-induced permeability changes. In addition, it has recently been demonstrated that isolated fragments of the *Electroplax* electric organ can be used to quantitatively study acetylcholine-induced ion transport (J. P. Changeux, personal communication). This technique could be adapted to purified animal-cell membranes. Once the assay for depolarization is functional, the detailed pharmacology and kinetics of chemically induced depolarization could be studied. Then, similar techniques could be used to study catecholamine uptake and release and,

finally, the coupling between acetylcholine depolarization and neurotransmitter release. (See below.)

IV. Acetylcholine Receptor

Since the neuroblastoma tissue culture line can be induced to generate an action potential by the extracellular iontophoretic application of acetylcholine, it necessarily has the acetylcholine receptor. The molecular mechanism which is responsible for acetylcholine-induced depolarization remains obscure; the neuroblastoma could clarify matters in at least two ways. These are the possible isolation of the acetylcholine receptor and, of more interest to the understanding of excitable membrane function, the selection and characterization of mutant cell lines blocked in their ability to respond to acetylcholine and others lacking the (or having an altered) active sodium-potassium pump.

There appear to be two philosophies regarding the characterization and isolation of the acetylcholine receptor. Several investigators have attempted to affinity-label the receptor protein (Changeux, Podleski, and Wofsy, 1967; Silman and Karlin, 1969), with relatively little success. Part of the difficulty is due to the heterogeneity of the cell population being labeled, which can be overcome by the use of cloned tissue culture lines. The other major difficulty, the relative lack of specificity of the highly reactive labeling reagent, is present in both systems. On the other hand, few investigators have tried directly to isolate the receptor using a binding assay for acetylcholine (O'Brien and Gilmour, 1969). This method has the distinct advantage of being able to work with a functional protein as opposed to an inactive affinity-labeled molecule. The techniques of equilibrium dialysis, and particularly the membrane filter assay (Jones and Berg, 1966; Riggs, Bourgeois, Newby, and Cohn, 1968; Schubert, Roman, and Cohn, 1970), would be most applicable. Cytoplasmic membranes are readily prepared, and these could be gently taken apart by a unique nonionic detergent, Nonidet P40 (Particle Data Laboratory Limited, Elmhurst, Illinois). This detergent has been shown to enhance the activity of several membrane-bound enzymes (Y. S. Choi, personal communication), and it is possible to do immune precipitations and hapten-antibody equilibrium dialysis in the presence of 1 percent detergent (Schubert, unpublished observation). Thus, by controlled lysis with detergent, followed by the fractionation of acetylcholine-binding activity in the presence of esterase inhibitors, it may be possible to isolate the pharmacological receptor. Reconstitution of the dissociated membrane may lead to a better understanding of both structure and function.

The isolation of the receptor could be facilitated by a comparison with cells which lack a functional receptor – by looking for a structural difference between mutant and wild type. The receptor-negative mutants may be isolated in the following manner. Acetylcholine or one of its functional analogues would be coupled to bovine serum albumin or any other suitable carrier. A population of mutagenized cells would be grown up and various concentrations of the conjugated acetylcholine added to the suspension. Antiserum prepared against the carrier would be added, followed by the addition of complement. In this manner, cells having the acetylcholine receptor would bind the hormone-protein conjugate and be lysed when anti-protein and complement were added. The survivors would be grown up and the selection procedure repeated many times until the survival was sufficiently high to indicate the existence of mutants. The cells would then be cloned and individual clones tested both electrophysiologically and directly for the binding activity.

A similar selection procedure may be used to select mutants effecting active sodium-potassium transport. Ouabain specifically blocks active transport at an apparently external membrane site in nervous tissue (reviewed by Whittam, 1967). The same selection procedure as above may be functional, this time coupling ouabain

to the carrier. Acetylcholine esterase mutants would similarly be selected by coupling esterase-specific reagents, such as eserine, to the carrier molecules.

It should be pointed out, however, that the above selection procedure depends on the ability of the conjugated hormone or blocking agent to function normally. If the initial protein conjugates are not functional, it is relatively easy to couple the pharmacological agents on less conformationally rigid antigenic carriers such as polyamino acids or polysaccharides.

An alternative possibility for selecting both acetylcholine-receptor-negative mutants and ouabain-insensitive mutants is the following. It has been shown that cells grown in certain levels of ouabain are lysed by this reagent, presumably due to an alteration in their active transport mechanism (Schubert, unpublished observation). If cells were mutagenized and grown in a concentration of ouabain which limits growth 80 percent to 90 percent until the inhibition is partially overcome, followed by increasing the concentration of ouabain, mutants resistant to the drug may eventually be selected. A similar procedure has been successfully used to select hydrocortisone-resistant lines of a mouse lymphoma (Baxter, Harris, Tomkins, and Cohn, 1971).

The same technique may also be used to select against cells with the acetylcholine receptor. If cells were grown under conditions of *partial* growth inhibition – a 10 percent decrease in growth rate – by ouabain, it follows that any alteration in the delicately balanced active-transport-mediated permeability could be lethal or growth-inhibitory to the cells. Thus, if cells were grown under these conditions in the presence of a cholinergic agonist such as decamethonium, the cells which respond (those with an active acetylcholine receptor) would be selectively killed, selecting for uneffected receptor-negative mutants. This effect should be blocked by antagonists, such as *d*-turbocurarine and flaxedil, thus defining the specificity of the selection procedure. Since the pharmacology of the receptor on this neuroblastoma has not yet been studied in detail, a large number of different agonists and antagonists will be tested.

Once mutants of the acetylcholine receptor, the acetylcholine esterase, and the ouabain-sensitive adenosine triphosphatase-activated sodium-potassium pump are isolated, the functional relationships between these processes could be studied and a number of *specific models* of nervous function tested. For example, it has been proposed that the acetylcholine receptor and the acetylcholine esterase are necessary for the conduction of an action potential (Nachmansohn, 1966) and that the acetylcholine receptor and the acetylcholine esterase may be the same molecule (Changeux, Leuzinger, and Huchet, 1968). Both models require that the receptor be coupled to an ion transport mechanism. Thus, if a mutant were isolated which lacked the acetylcholine receptor, but were still able to conduct an action potential, Nachmansohn's argument would be weakened; similarly, a mutant lacking the esterase but still able to be depolarized by acetylcholine would rule out Changeux's model. By scoring for the pleotropic loss of several unique functions following selection against one, a great deal of insight could be gained into both membrane structure and function in nervous tissue. It is doubtful that physical and chemical characterization of membranes alone can generate this kind of information any more than the details of macromolecule biosynthesis and regulation in bacteria could be understood without the aid of mutants.

Finally, it should be pointed out again that this neuroblastoma cell line is tetraploid, a fact which makes the selection of mutants more difficult, but far from impossible. Mutants derived from tetraploid plasmacytoma cell lines have been described and studied in detail (Schubert, Munro, and Ohno, 1968; Schubert and Horibata, 1968; Schubert and Cohn, 1968; Schubert and Cohn, 1970). Furthermore, it has been possible to select large numbers of mutants from tetraploid cells which are defective in surface antigens by a cytotoxic selection procedure similar to that described above (R. Hyman and M. Cohn, personal communications). It is, however, like-

ly that any selection on polyploid cell lines may generate a disproportionally high number of trans-dominant regulatory-type mutations, a possibility which makes their study *no* less interesting.

V. Storage, Release, and Uptake of Catecholamines

It is known that the catecholamine neurotransmitters are stored in dense-core vesicles localized in the presynaptic nerve endings of the sympathetic nervous system (reviewed by Iverson, 1967). It is also known that, following release, the adrenergic transmitter is inactivated by uptake into the presynaptic nerve ending from which it originated. The molecular mechanisms of catecholamine uptake, storage, and release are for the most part unknown, primarily because of the complexity of the tissues studied (Iverson, 1967). The neuroblastoma tissue culture line synthesizes and accumulates catecholamines, and the cells also contain dense-core vesicles which are morphologically similar to those thought to contain norepinephrine. In addition, these vesicles are frequently found concentrated in regions of cell-cell contact which are suggestive of chemical synapses (Schubert, Humphreys, Baroni, and Cohn, 1969). It follows that this tissue culture line may be an ideal system to investigate transmitter release and its integration with axonal depolarization.

To establish that depolarization leads to neurotransmitter release, a Millipore chamber would be built which would contain the cells and a pair of electrodes and, at the same time, allow a steady stream of medium to pass around the cells. The outflow of this system would then be attached to a fraction collector. Since it is possible to induce cell depolarization and norepinephrine release in tissue slices by passing a low-frequency 5 V ac potential across a tissue culture dish (Baldessarini and Kopin, 1966), by using a similar mechanism catecholamine release should be demonstrable in these cells. In addition, if an assay for chemical depolarization is successfully developed (Section III), it should be possible to devise a membrane assay for both catecholamine uptake and release. For example, cells would be "loaded" with ^{14}C-norepinephrine or a radioactive nondegradable false transmitter, depolarized with an agonist, and the rate of release of transmitter studied by stopping the reaction by collecting the cells on a Millipore filter. Similar experiments could be used to study catecholamine uptake. The numerous pharmacological agents which affect storage and release could then be studied initially at this macroscopic level, followed by a more rigorous definition of their modes of action. Perhaps some of these results would prove relevant to the natural mode of release.

Probably a more direct approach would be the isolation and characterization of the synaptic vesicles (Whittaker and Sheridan, 1965). Once purified, questions could be asked relative to the effect of membrane depolarization on the release of transmitter from isolated vesicles and whether or not individual vesicles are capable of catecholamine synthesis and uptake.

The derivation of the limiting membrane of the vesicles and possibly the mechanism of transmitter release could be investigated if it were possible to prepare antisera which recognize only vesicle membrane or the unique protein of unknown function which is found associated with adenosine triphosphate inside purified norepinephrine-containing vesicles (Iverson, 1967). There are two models which can be used to explain transmitter release; by the use of specific anti-vesicle sera one of these models could be ruled out. Either the vesicles internally release their contents which are then channeled to the outside, or the vesicles fuse directly with the presynaptic membrane, releasing their contents directly into the synaptic cleft, and the vesicle membrane becomes incorporated into the limiting cellular membrane. The latter may make sense if vesicle membranes could contribute to specificity, for it has frequently been suggested that repeated firing stabilizes nerve networks (Cohn, 1970). A necessary consequence of firing is the release of transmitter (and perhaps the

vesicle protein) into the cleft. The vesicle-membrane fusion may reinforce the connection, as may the protein of unknown function contained in the vesicle. In this context, it may be of interest to see if synapses (electrical or chemical) are formed more rapidly in cultures which are frequently depolarized by alternating current.

One type of experiment would be the following. Two groups of ^{3}H-leucine-labeled cells would be prepared, and one would be electrically depolarized for 30 minutes. At the end of this time, antiserum directed against synaptic vesicle membrane, and another antiserum against vesicle protein, would be added to separate aliquots of washed cells and cell supernatants. A cytotoxic test would be done with the cells, looking for killing by the two types of anti-vesicle sera. If the depolarized cells are killed by the antisera and not the control, it would indicate that vesicle or vesicle contents are extracellularly bound after repeated firing of the cell, thus supporting the vesicle-membrane-fusion model. Similarly, the release of vesicle protein into the cell supernatant would be assayed by serological precipitation and acrylamide electrophoresis (Schubert, 1968). It would also be worth looking for other release proteins by running the entire cell supernatant on gel electrophoresis and comparing the two types of cells. This type of experimental approach could also be used to look at other cell functions following repeated firing.

In addition to looking at normal function, it should be possible to select mutants which lack synaptic vesicles and possibly the ability to synthesize catecholamines. It has been shown that 5- and 6-hydroxydopamine selectively destroy sympathetic nerve endings in vitro (Tranzer and Thoenen, 1968; Thoenen and Tranzer, 1968), with the concomitant loss of tyrosine hydroxylase activity (Mueller, Thoenen, and Axelrod, 1969). The effect of these two compounds on cell viability will be tested with respect to dosage and time required for killing. After a suitable concentration of the dopamine derivatives is determined, resistant cells will be selected in suspension, and possibly on agar plates containing the catecholamine. Clones of resistant lines will then be characterized with respect to catecholamine synthesis, uptake, and release, and also synaptic vesicle synthesis. If it were possible to isolate lines lacking vesicles but still able to synthesize and release catecholamines, the controversies relative to the role of synaptic vesicles would be partially solved.

VI. Cyclic AMP

After reviewing the current literature, it appears that no grant request would be complete without a section on adenosine 3′,5′-cyclic monophosphate (cAMP), so in this light we will make the following proposals. It is clear that cAMP plays a central role in the regulation of a large number of membrane-mediated, hormone-dependent processes (reviewed by Butcher, Robinson, and Sutherland, 1970). In addition, increasing evidence indicates that cAMP may promote nervous transmission by facilitating the release of neurotransmitters (Goldberg and Singer, 1969). There are thus two points in the neuroblastoma system where cAMP may be involved.

The first of these is the membrane-mediated induced differentiation of the neuroblast to neuron. Since cAMP mediates many hormone-dependent membrane phenomena, it is a good candidate to be involved in the induction of differentiation. This could be tested by examining the effects of cAMP, dibutyl cAMP, and the methyl xanthines on the process of differentiation and also by looking for changes in the levels of intracellular cAMP and the specific activity of adenyl cyclase under conditions leading to differentiation. If differentiation can be induced or enhanced by mimicing the adenyl cyclase system, then the cAMP effect will be examined in detail, particularly with respect to several proteins which appear to be induced by conditions leading to differentiation. The microtubule protein appears to be the most accessible in this group, and the control of its synthesis could be examined in a cell-free system. If mutants are found which are unable to differentiate (Sec-

tion I), the possibility of a genetic lesion at the cAMP step will be examined. In this manner, some insight into the mechanism by which a cell handles a surface (tactile) stimulus (a process which is clearly of great importance to tissue formation and differentiation) may be gained.

The second area of interest is the role of cAMP in neurotransmitter release, for the neuroblastoma offers a unique opportunity to study this process. To determine whether or not cAMP is involved in release, cells would be "loaded" with radioactive norepinephrine or a nonmetabolizable false transmitter and the rate of transmitter release (both spontaneous and induced) studied in the presence of cAMP, dibutyl cAMP, and the methyl xanthines by methods described in Section III. If cAMP is involved, the rate of release should be facilitated by these compounds (Goldberg and Singer, 1969). Once facilitation is observed in the whole-cell system, then it should be possible to take apart the cell and ask specific questions regarding the integration or coupling of acetylcholine depolarization and transmitter release. For example, do acetylcholine and other agonists directly affect the specific activity of adenyl cyclase? Does cAMP induce the release of catecholamines from purified synaptic vesicles or membrane fragments?

VII. Complementation

The observation that the mouse neuroblastoma can undergo *reversible* differentiation (Schubert, Humphreys, de Vitry, and Jacob, 1971), along with the possibility of inducing somatic cell hybrids (Harris and Watkins, 1965), offers the opportunity to study the complementation between cells at various stages of phenotypic expression. For example, attached cells at various stages of differentiation could be fused by Sendai virus with rapidly dividing undifferentiated cells whose nuclei are marked with ^{3}H-thymidine. After fusion, the unattached, undifferentiated cells would be washed away and the attached cells placed under conditions where mitosis is inhibited – low serum, 5-fluorodeoxyuridine, or cytosine arabinoside (Schubert, Humphreys, de Vitry, and Jacob, 1971); then, specific questions could be asked regarding the complementation of functions at the level of a *single cell.* For example, are the electrophysiological properties and acetylcholine sensitivity of the fused cell similar to the undifferentiated or differentiated parent? This could be answered by recording from a large number or cells and marking them by the iontophoretic injection of dye, followed by radioautography to score for the fused cells. In addition, if a number of mutants are selected which lack a given function (the acetylcholine receptor), complementation groups could be defined in the manner described above, without the need for growing out clones from fused cells, which is, in most cases, a very difficult task.

VIII. Other Cell Lines

It is planned that, during the tenure of this research proposal, the types of investigations outlined above can be extended to several other tissue culture lines, including those of muscle, adrenal chromaffin cells (which share many properties of the mouse neuroblastoma), and other cells of nervous tissue origin. In conjunction with Dr. M. Cohn (The Salk Institute), attempts are being made to induce brain tumors in a large number of mice by means of a wide spectrum of carcinogens. (See, for example, Ivankovic and Druckrey, 1968.) If successful, the induced tumors will be adapted to tissue culture by the methods used for the initial mouse neuroblastoma and characterized as described above. In addition, attempts will be made to establish embryonic cell lines in culture by transforming *specific* embryonic tissues in vitro, either with viruses (Dulbecco, 1969) or chemically (Heidelberger, 1964). This approach was successful with embryonic skeletal muscle (Yaffe, 1968), and there is no reason why it could not be extended to the nervous system.

IX. Tentative Time Schedule

The following[3] time schedule is based on the assumption that three postdoctoral fellows, a research technician, and the principal investigator are involved full time in the research the first year and that an additional postdoctoral fellow and an additional research technician are added during the second year.

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[3] The time schedule that followed this paragraph in the original proposal is not reprinted here.

[4] Throughout this References section, an asterisk preceding an entry denotes the reference was in press or submitted for publication when the application was written.

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* * *

THE RESEARCH GRANT BUDGET: PREPARATION AND JUSTIFICATION IN RELATION TO THE PROPOSED RESEARCH

by James M. Pike* and Steven C. Bernard†

Although the research grant budget is usually prepared after the text of a proposal has been completed, it is nonetheless an integral part of a research grant application and must clearly reflect resources needed for the proposed project. Experienced grant applicants know that the members of the staff of the granting agency and the reviewers expect to find a clear and justifiable relation between the proposed project and the requested budget. Without such a total presentation, there is little chance that the budget will be approved as requested.

The preparation of the budget should be approached as a two-part exercise. First, funds

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are listed for specified categories. For the application used by the National Institutes of Health (NIH), the applicant fills in descriptors and dollar amounts on a printed budget form with specified categories.* Secondly, detailed and concise justification or explanation is provided for each category of funds requested. The need for a carefully considered justification for the budget cannot be emphasized too strongly. Those who have observed or participated in the review of research grant applications have repeatedly heard the reviewers state such criticisms as: "the need for that item of equipment is not clear," "there is no justification for that number of animals," or "this project would not seem to require that much participation of technical staff." Such statements are usually the result of an inadequate justification for the requested budget. The applicant should know best what the budgetary needs are for the project proposed and, if asked, could usually explain the reason for each amount requested. The review of most research grant applications, however, does not include an on-site visit.[1] Consequently, the merit of a research grant application must be evaluated almost solely on the basis of the application, without any opportunity for discussions between the applicant and the reviewers.[2,3] While a well-written presentation of the scientific protocol may serve indirectly to justify the requested budget, the need for a separate, complete, and concise explanation of the requested budget facilitates the review and enhances the likelihood that the budget will be recommended for funding in the amount requested.

The extent of clarification that accompanies the budget is best judged by the applicant. The detail that might be included as part of the wirtten clarification, however, depends on a number of considerations, such as the clarity and completeness of the text of the application, the kinds and quantity of equipment and supplies already available, and the prospective judgment of future needs as the research progresses. The content of the clarification of the budget should also be developed with a constant awareness of the time the reviewers have to read each application. Since a large number of applications have to be reviewed at a meeting of the review group, the considerate – and wise – applicant will provide readily accessible, concise, and clear justification for the budget.

*The printed "Detailed Budget for First 12-Month Period" is provided as page 3 of the "Application for Public Health Service Grant or Award" (Form NIH 398). An example of this page of the NIH application form was presented in *Grants Magazine*, Vol. 1, No. 2, p. 175.

Justification for the First Year

The explanation of the budget should be prominently entitled "Justification for the Budget," or "Explanation of the Budget," and it should have the same sequence of items or budgetary categories as on the printed budget page. A simple and direct format enhances the usefulness and impact of this section. Each cost category, such as personnel, equipment, supplies, and travel, should clearly stand out as subsections so that the reviewers may quickly and easily find the needed information. In an example of such a format, we have presented below examples of typical questions that reviewers ask themselves as they assess the appropriateness of the requested budget. Categories for which funds are requested only rarely, such as for alterations and renovations and for patient care, usually require specific guidance from the anticipated awarding component of the NIH and have therefore not been included in the following list.

Personnel

- Are the number of professional and nonprofessional staff consistent with the effort required by the research protocol?
- Does the amount requested for personnel to be appointed reflect anticipated beginning dates for such appointments?

- Are the requirements for some portions of the research protocol so extensive that they justify what might otherwise be considered an inflated request for personnel?
- Is the time or effort allocated for each person consistent with other concurrent or pending efforts with which each may be involved?
- Does the budget reflect the time or effort for those who would be working on the project but who would receive total or partial remuneration from other sources of support?
- Does the budget reflect an appropriate request for fringe benefits?
- Does the institution allocate fringe benefits to indirect costs or to direct costs?
- Does the request reflect midyear salary increases that could be anticipated when the application was prepared?

Consultant Costs

- Who are the consultants?
- With what institutions are the consultants affiliated?
- Are the consultants so well known that biographical sketches are not needed?
- What portions of the project require advice from consultants?

Equipment

- Has it been determined whether these items are already available in the institution's inventory of unused equipment?
- Has it been determined whether these items are available in nearby laboratories and could be shared?
- Would it be more economical to rent any of the items rather than purchase them?

Supplies

- Are the major subcategories of supplies identified, such as animals, chemicals, and glassware?
- Where applicable, are unit prices shown?
- How has the rate of usage been determined?

Travel

- Is the amount of and reason for the anticipated travel consistent with the goals of the research project and with the amount of effort that the investigator will devote to the project?
- Has special justification been provided for any foreign travel requested?

Other Expenses

- Have maintenance costs for laboratory animals been included? Have these costs been estimated on the basis of the guidelines for the care of laboratory animals?
- Does the project require purchased services from third parties or other institutions? If so, has the need for third-party indirect costs been anticipated?

The Future Years

The application used by the NIH provides the opportunity to request up to seven years of support.* Development of the budget for future years of research support requires considerable foresight in predicting research results and probably a certain amount of clairvoyance. While many granting agencies have the authority to adjust budgets upwards to accommodate unforeseen needs, fiscal constraints often preclude the provision of exigency funds. For this reason, carefully and thoughtfully developed budgets

*The printed "Budget Estimates for All Years of Support Requested from Public Health Service" is provided as page 4 of the "Application for Public Health Service Grant or Award" (Form NIH 398). An example of this page of the NIH application form was presented in *Grants Magazine*, Vol. 1, No. 2, p. 176.

for future years are clearly advantageous to the applicant. The budgets for future years should also be accompanied, where appropriate, by written justification or explanation.

For the assessment of financial needs in future years, the simple percentage-based extrapolation of inflation-prone categories such as personnel or supplies will only partially suffice. When it is possible to do so, the investigator should estimate as accurately as possible the actual resources needed in future years. These budgets should reflect not only a reasonable and appropriate extension of the request for the first year but also the changing needs of the research project. For example, justification for the budgets for future years should reveal the rationale for increased or decreased amounts in the different categories. How have cost-of-living adjustments or promotions as mandated by institutional policy been reflected? Will the need for personnel increase or decrease in future years, and are the reasons clear? In relation to anticipated progress in the research, is there a need for a new and specific item of equipment in a future year, and has that been justified?

In summary, the judicious preparation of a budget for a research grant proposal should include an accompanying section that clearly and concisely justifies the funds requested for each category. Even though an applicant may feel that the text of the proposal provides adequate justification for the budget requested, the relation between the budget and the proposed project may not be readily apparent to the reviewers. Since the success of the proposed research project is dependent on adequate resources, the applicant should make certain that all information needed for the most advantageous review is readily available and that nothing is left to chance.

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